THE EFFECT OF GLUCAGON ON UREAGENESIS FROM AMMONIA BY ISOLATED RAT HEPATOCYTES

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Summary: Glucagon, at a maximally effective concentration of 1 μM, stimulated by 35% the rate at which rat hepatocytes synthesized urea from 10 mM NH_4Cl in the presence of 10 mM ornithine. The rate at which citrulline accumulated in the incubations was relatively unchanged by the presence of glucagon.

Mitochondria isolated from glucagon treated hepatocytes were observed to synthesize citrulline from 10 mM NH_4Cl and 10 mM ornithine more rapidly than did mitochondria isolated from untreated hepatocytes.

The role of the intracellular malate concentration in the regulation of the rate of urea synthesis, and the changes observed in the cellular content of malate in response to glucagon are discussed.

Introduction: Studies aimed at elucidating the effects of glucagon on mammalian hepatic metabolism have centered largely on the ability of the hormone to stimulate gluconeogenesis (1-3). The effect of glucagon on another important metabolic pathway of mammalian liver, that of urea synthesis, has not been as extensively investigated. Glucagon has been shown to increase urea production from amino acids by the perfused liver (4), apparently by increasing the rate of amino acid degradation and thereby increasing the amount of substrate available for urea synthesis. This report will demonstrate that glucagon is also capable of stimulating the rate of urea production by isolated hepatocytes in the presence of excess substrate (10 mM NH_4Cl), indicating that a rate limiting step in the synthesis of urea from ammonia is subject to hormonal control by glucagon.

During the preparation of portions of this material for publication (5) it was noted that Yamazaki and Graetz reported an increased rate of citrulline production by mitochondria isolated from rats treated with glucagon (6). The data reported here substantiate their observation and extend it to the isolated hepatocyte system. The use of the hepatocytes eliminates the possibility of extra-hepatic effects of the hormone being responsible for the observed effects on mitochondrial citrulline production.

Methods: Rat liver cells were prepared from 200-300 gm male rats (Sprague-Dawley, Hilltop Lab Animals, Scottsdale, Pa.) which had been starved for 18-24 hours. The method of cell isolation was essentially that of Berry and Friend

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Table 1. Effect of Glucagon on the Rates of Ureagenesis and Citrulline Accumulation by Isolated Hepatocytes. Isolated hepatocytes were incubated with 10 mM NH_4Cl and 10 mM ornithine either in the presence or absence of 1 μM glucagon.

	nmoles/min · mg^{-1} DNA	
	No glucagon	1 μM glucagon
Urea production	192 ± 15	261 ± 15*
Citrulline accumulation	67 ± 5	74 ± 5*

*different from control by paired t-statistic, $p < 0.001$, $n = 15$

(7) as modified by Krebs (8). Hyaluronidase was omitted. The final cell pellet was suspended in 13-17 volumes of Krebs-Henseleit-albumin (8) and two milliliters of the cell suspension were added to each incubation flask which contained 2 ml of Krebs-Henseleit-albumin and the substrates and hormones to be tested. Incubations were carried out in duplicate in 30 ml Nalgene bottles and were stopped at timed intervals by addition to $HClO_4$. The amount of urea and citrulline produced in the incubations were assayed as previously described (9). DNA was determined by the diphenylamine method (10). Glucagon and bovine serum albumin (Fraction V) were obtained from the Sigma Chemical Co. Glucagon was also obtained by a generous donation from the Eli Lilly Co.

Preparation of isolated mitochondria from hepatocytes: Hepatocytes were incubated either with or without 1 μM glucagon for 10 minutes at 37°. The cells were then harvested and washed with cold Krebs-Henseleit-albumin. The cell pellets were then washed with cold homogenization buffer (0.3 M mannitol; 3.4 mM Tris, pH 7.4; 1 mM EGTA) and resuspended in 4-5 ml of the homogenization buffer for homogenization with 20 strokes of a Teflon pestle Potter-Elvejhem homogenizer. Mitochondria were isolated by standard centrifugation techniques (11) and the pellet washed once before the mitochondria were resuspended for use in the incubations. Protein was determined by the Biuret method (12).

Results: When isolated hepatocytes from starved rats were treated with a maximally effective dose of glucagon (1 μM) the rate of urea synthesis from 10 mM NH_4Cl in the presence of 10 mM ornithine was increased an average of 35% relative to the control rate (Table 1). The rate of citrulline accumulation remained relatively unchanged in the presence of glucagon. The rates of urea synthesis observed in this study are similar to those reported in the literature for the production of urea from ammonia by isolated rat hepatocytes (9) and are also comparable to the rates of urea synthesis observed by other workers from the perfused liver under similar conditions (13, 14). The concentration of glucagon which was observed to produce half-maximal stimulation of urea synthesis was 50 nM. Dibutryl-cAMP (0.1 mM) and epinephrine

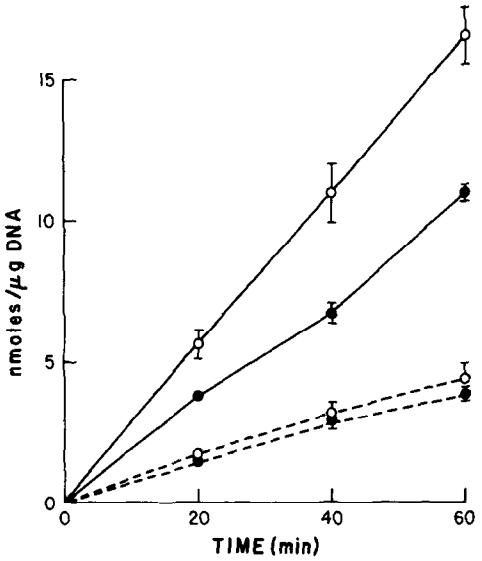

Figure 1. The effect of glucagon on urea synthesis and citrulline accumulation by isolated hepatocytes. Cells were incubated for the designated time with 10 mM NH_4Cl and 10 mM ornithine in the presence (○) or absence (●) of 1 μM glucagon. Each point is the mean of three observations, with the vertical bar representing the SEM.

(10 μM) were also observed to stimulate urea synthesis to the same extent as did 1 μM glucagon (data not shown).

Fig. 1 shows the time course of urea synthesis and citrulline accumulation by hepatocytes incubated with 10 mM NH_4Cl and 10 mM ornithine. Rates of urea synthesis were linear both in the presence and in the absence of glucagon and it is apparent that there was no significant lag in the onset of the glucagon mediated stimulation of urea synthesis. Citrulline accumulation continued in a linear manner for the entire 60 minute incubation period.

Mitochondria isolated from hepatocytes which had been treated with glucagon (1 μM) synthesized citrulline at a greater rate than did mitochondria isolated from hepatocytes which had not been treated with glucagon (Table 2). Rates of citrulline production by mitochondria from both control and glucagon treated hepatocytes were linear throughout the incubation period of 10 minutes (Fig. 2). When mitochondria were incubated in the absence of ornithine, radioactivity in acid stable products was only 3-5% of that found in the presence of ornithine and was not greater in incubations of mitochondria from glucagon treated hepatocytes. This ornithine independent fixation of radioactivity was subtracted from the total acid stable radioactivity in each experi-

Table 2. Effect of Glucagon Treatment of Isolated Hepatocytes on the Rate of Citrulline Production by Mitochondria Prepared from Isolated Hepatocytes. Mitochondria were incubated in triplicate as described below. Rates were statistically different by Students t-test as indicated by: (*), $p < 0.05$; (**), $p < 0.01$; (***), $p < 0.005$.

	Citrulline Production nmoles/min per mg protein ± SEM		
	no glucagon	+ glucagon (2 μM)	% of Control
Trial 1	44.37 ± 0.09	47.63 ± 0.24*	107
Trial 2	40.55 ± 0.90	45.33 ± 0.27**	112
Trial 3	20.15 ± 0.17	23.73 ± 0.55***	118

Incubation flasks contained (final concentrations) KLL, 80 mM; Tris, pH 7.4, 25 mM; $KHCO_3$, 10 mM; KH_2PO_4, 10 mM; NH_4Cl, 10 mM; ornithine, 10 mM; succinate, 10 mM; rotenone, 0.5 μg/ml and [^{14}C]$NaHCO_3$, 0.8 μCi/ml.

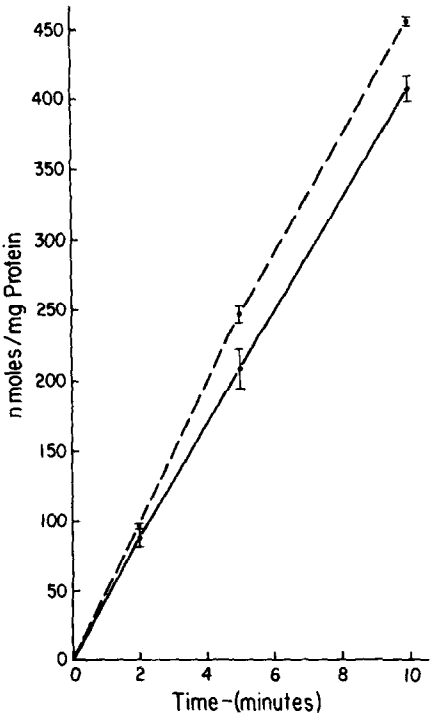

Figure 2. Citrulline production by mitochondria isolated from control and glucagon treated hepatocytes. Mitochondria were incubated as described for Table 2. Broken line represents mitochondria from glucagon treated cells, vertical bar represents the SEM.

ment. Direct treatment of the mitochondria with glucagon (1 μM) or with cyclic-AMP (0.1 mM) did not result in significant changes in the rate of citrulline production (data not shown).

Discussion: The observations reported here indicate that glucagon is capable of stimulating a process which is rate limiting to the synthesis of urea from saturating levels of ammonia by isolated rat hepatocytes. The absence of any significant lag in the establishment of the glucagon stimulated rate of urea synthesis indicates that protein synthesis is probably not a required step in the mechanism of glucagon action on urea synthesis. This very rapid effect on ureagenesis is similar to the time-course observed for glucagon stimulation of gluconeogenesis (1-3).

In this study ornithine was included in the incubation medium to provide an adequate supply of urea cycle intermediates. Under these conditions, citrulline was observed to accumulate in the incubations, suggesting that a rate limiting step in urea synthesis existed between citrulline and urea. Since arginine does not accumulate in these incubations (unpublished data, Triebwasser and Freedland), the rate limiting step is actually between citrulline and arginine. Glucagon did not alter the position of the rate limiting step since the presence of the hormone did not appreciably change the rate of citrulline accumulation.

It has been suggested that in hepatocytes isolated from starved rats the utilization of citrulline is limited by the availability of aspartate in the cytosol (9, 15). The observed accumulation of citrulline (Table 1) is consistent with a limitation in the availability of aspartate for the conversion of citrulline to arginine. Meijer, et al. (14) have further suggested that in the absence of gluconeogenic precursors it is actually the cytosolic concentration of malate which limits the rate at which mitochondrial aspartate formation and efflux to the cytosol occur. It is therefore of interest that glucagon treatment of hepatocytes resulted in an increase in the steady state intracellular malate content from 8.3 ± 1.5 nmoles/mg DNA to 11.3 ± 1.7 nmoles/mg DNA (mean, ± S.E.M., n = 5; significantly different by the paired t-statistic, $p < 0.02$), concomitant with an increase of 40% in the rate of urea synthesis. In these experiments, the intracellular malate content of the hepatocytes was measured utilizing a silicone oil-centrifuge stop technique (15), coupled with a fluorometric modification of the assay for malate (16). The observed increase in the steady state intracellular content of malate in response to glucagon correlated very well (mean value of $r = 0.93$ for five observations) with the observed increase in the rate of urea synthesis (unpublished data, Triebwasser and Freedland). These results suggest that

glucagon treatment increases the rate of flux through the urea cycle in hepatocytes by causing an increase in the intracellular malate concentration. This effect may be mediated through the stimulatory effect of glucagon on pyruvate carboxylation (17).

The observation that glucagon treatment of hepatocytes results in a stable increase in the rate at which isolated mitochondria will synthesize citrulline indicates that glucagon action on the cell alters in a stable manner a reaction critical to determining the rate of mitochondrial citrulline production. However, since citrulline accumulates in the hepatocyte incubations, an increase in the ability of the mitochondria to synthesize citrulline as a result of glucagon treatment would not be expected to account for the increased rate at which urea was synthesized by the hepatocytes in the presence of glucagon. Glucagon treatment of the intact rat (6) causes a much larger stimulation of mitochondrial citrulline production than does direct treatment of the hepatocyte with glucagon (Table 2). This fact suggests that a portion of effect observed when glucagon is administered directly to the rat (6) may well be due to indirect effects mediated by extra-hepatic actions of the hormone.

The mechanism by which glucagon treatment causes an increase in mitochondrial citrulline production is not clear, however, this effect could involve an increase in the mitochondrial content of N-acetylglutamate, a known effector of carbamyl phosphate synthetase (18).

The data in this study provide information which is valuable in understanding both the factors which determine the rate of ureagenesis from ammonia by isolated hepatocytes and also the mechanisms by which glucagon alters cellular metabolism.

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